Toxicity evaluation of 24 anesthetic compositions in CaCO-2 cell line (from epithelial human tissue)

1.- OBJETIVE

The objective of this research is to demonstrate the synergistic effect in the decreasing of the adverse effects when we use a combined anesthetic with Lidocaine (L), Prilocaine (P) and Tetracaine (T), compared with each ingredient alone and in the same total concentration.

We take into account the following:

- In order to have a valid comparison, the compositions have to maintain the same total amount of anesthetic in each composition which is going to be compared.
- Testing different ranges of concentration according to claims of the patent application.

The 24 anesthetic compositions to be tested are the following:

1) Lower concentration range which is claimed: 0.5L + 0.5P + 0.5T (1.1), compared with:

1.2) 1.5L 1.3) 1.5P 1.4) 1.5T

2) Higher concentration range which is claimed: 5L + 5P + 8T (2.1), compared with:

2.2) 18L 2.3) 18P 2.4) 18T

3) Composition 1.5L + 1.5P + 4T (3.1), compared with:

3.2) 7L 3.3) 7P 3.4) 7T

4) Ternary composition which keeps the same proportion of each agent as the above mentioned point (1.5L + 1.5P + 4T), but with a total sum of the anesthetic similar to EMLA and AMLI (5 parts of total anesthetic). In the same way two other ternary combinations will be tested which keep the total sum of anesthetic equal to EMLA and AMLI (5 parts):

4.1) 1.07L + 1.07P + 2.86T;

4.2) 1.5L + 1.5P + 2T; and

4.3) 1.5L + 2P + 1.5T, compared with:

4.4) 5L 4.5) 5P 4.6) 5T 4.7) 2.5L + 2.5P (EMLA)4.8) 2.5L + 2.5T (AMLI)

5) Composition 1.5L + 1.5P + 8T (5.1) compared with:

5.1) 11L 5.2) 11P 5.3) 11T

Concentrations of each anesthetic are in % w/w.

2. MATERIALS AND METHODS

In order to compare the above mentioned we study the cytotoxicity of these 24 anesthetic compositions on CaCO-2 cells (human epithelial cells) by determining cell viability through WST-1. The study was carried out 24 hours after the treatment with the different anesthetic compositions, and four independent tests were done in triplicate.

The cytotoxic effect of a compound is determined by evaluating the percentage of cell death which the compound produces in comparison with a group of control cells which have not been treated. In order to do this, cell viability is measured by determining metabolic activity through a WST-1 (Roche) test. This method is based on the capacity of cells to obtain the energy necessary in order to continue their functions and to produce cell growth. For this reason, cells which are metabolically active (alive) reduce tetrazolium salts to formazan by means of the enzyme succinate-tetrazolium reductase (of the mitochondrial respiratory chain). The resulting formazan can be detected colourimetrically (see Figure 1). In contrast, this reaction does not occur with damaged or dead cells.

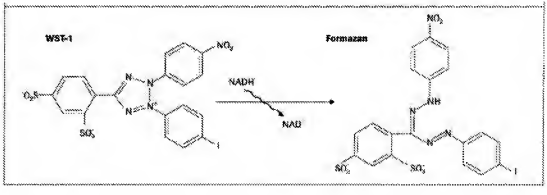


Figure 1. Chemical reaction produced by the enzyme succinate-dehydrogenase of the mitochondrial cell chain. Formation of formazan from WST-1.

In the first instance, a preliminary test was carried out in order to determine the optimum working concentration which would allow differences to occur in the cytotoxicity of the different anesthetic compositions, as well to verify that the excipient used would not, itself, produce toxicity. A first test was carried out in which the anesthetic compositions were diluted 1:10 in the growth medium. This caused the death of all the cells within a few hours after treatment. A second test was then carried out in which the anesthetic compositions were diluted 1:1000 in the growth medium. This provided appropriate results in order to be able to carry out a comparison between the anesthetic compositions.

Due to the viscosity of the anesthetic compositions, various tests were done in order to determine the procedure which would make reproducing the results the easiest. In the end, a dilution of 1:10 in cell growth medium was chosen. This was done 24 hours before the experiments were carried out in order to homogenize the dilution. At the time of the test, and after tempering the preparation, a second dilution of 1:100 in complete growth medium was carried out.

3. RESULTS

24 anesthetic compositions were tested on CaCO-2 cells in a final optimum dilution of 1:1000 of the cell medium. Toxicity was analyzed by measuring metabolic cell activity in (untreated) control cells and cells treated with the anesthetic compositions.

Composition	Samples	Medias	SD	SEM	P-value
EXCIPIENT		6	9	3	
0,5% L+0,5% P+0,5% T	**1.1**	2	8	2	REF.
1,5 % L	**1.2**	3	5	2	4,4E-01
1,5 % P	**1.3**	5	7	2	1,9E-01
1,5 % T	**1.4**	6	10	3	1,7E-01
5% L+ 5% P+ 8% T	**2.1**	23	16	5	REF.
18 % L	**2.2**	25	16	5	3,8E-01
18 % P	**2.3**	20	20	6	3,7E-01
18 % T	**2.4**	64	16	5	1,0E-06
1,5% L+1,5% P+4% T	**3.1**	42	17	5	REF.
7 % L	**3.2**	44	15	4	5,0E-01
7 % P	**3.3**	44	20	6	4,7E-01
7 % T	**3.4**	56	18	5	5,1E-02
1,07 % L+ 1,07 % P+ 2,86 % T	**4.1**	5	10	3	REF.
1,5 % L+ 1,5 % P+ 2 % T	**4.2**	8	8	2	2,5E-01
1,5 % L+ 2 % P+ 1,5 % T	**4.3**	10	11	3	1,6E-01
5 % L	**4.4**	21	11	3	8,7E-04
5 % P	**4.5**	12	7	2	3,3E-02
5 % T	**4.6**	17	9	3	3,5E-03
2,5 % L + 2.5 % P	**4.7**	17	7	2	2,6E-03
2,5 % L + 2.5 % T	**4.8**	23	7	2	4,6E-05
1,5% L + 1,5% P + 8% T	**5.1**	22	13	4	REF.
11 % L	**5.2**	22	10	3	4,5E-01
11 % P	**5.3**	16	12	3	1,2E-01
11 % T	**5.4**	50	17	5	2,5E-04

Table 1 shows the average percentage of toxicity caused by the anesthetic compositions on the CaCO-2 cells after 24 hours of treatment, compared to the (untreated) control cells. The averages given here are the averages of four independent tests carried out in triplicate. The standard deviation (SD) is given as well as the standard error of the mean (SEM). The p-value of the Student t-test is also given for each group of samples compared with the reference (REF) in each case. Differences are significant for p<0.05 (5.0E-02).

4. CONCLUSIONS

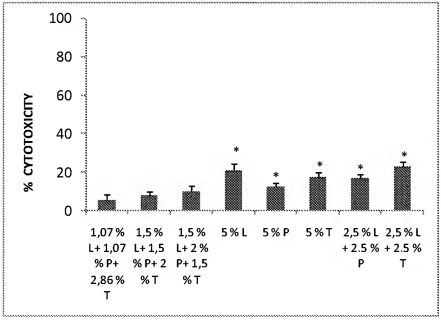
A cytotoxicity study was carried out on 24 anesthetic compositions in a cell culture: CaCO-2. The results indicate, in the first instance, that the excipient (in the dilution used) displays no toxicity in the cell cultures. The results obtained, therefore, are due entirely to the effects of the different anesthetics.

The result which stands out the most in the test carried out is the higher toxicity of the T anesthetic composition, while the L and P anesthetics presented lower toxicity, their results being quite similar.

In all cases the ternary combination of anesthetics (L,P,T) presents a lower toxic level than any of them separate. It is especially notable when we compare the ternary combination with T alone.

The most interesting results are found in the 4th assay (total anesthetic kept in 5 parts as EMLA and AMLI).

Below we can see the graphic representation of these results:



As we can see in the figure, in all studied cases, the ternary anesthetic combination presents a lower toxicity level compared with the rest of the samples.

From this significant information we can conclude that a three anesthetic combination (L, P, T) presents a lower toxicity in human epithelial cells than the anesthetic EMLA and AMLI in the same total sum of anesthetic (5 parts).